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The dissociation between upregulated endothelins and hemodynamic responses during polymicrobial sepsis

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Abstract

Polymicrobial sepsis is characterized by an early, hyperdynamic phase followed by a late, hypodynamic phase. Although studies have suggested that endothelins (ETs) contribute to the development of shock after a bolus injection of endotoxin, little is known about the role of ETs in the transition from the hyperdynamic phase to the hypodynamic phase of sepsis. To study this, male adult rats were subjected to sepsis by cecal ligation and puncture (CLP) followed by fluid resuscitation. Plasma levels of ET-1 and ET-2 were measured by radioimmunoassay at 2, 5, 10 h (i.e. the early stage of sepsis), and 20 h (late stage) following CLP or sham operation. Tissue levels of ET-1 and ET-2 were determined in the heart, lungs, small intestine, and spleen at 5 h after CLP or sham operation. In addition, preproendothelin-1 (precursor of ET-1) gene expression was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) at 5 h in the heart, lungs, small intestine, spleen, and liver. The results indicate that plasma levels of ET-1 and ET-2 were not different from values of sham groups at 2 and 20 h, but were significantly higher than the sham values at 5 and 10 h after CLP. While there were no significant increases in tissue levels of ET-1 and ET-2 at 5 h post-CLP, RT-PCR analysis indicates a significant upregulation of preproendothelin-1 gene expression in the heart, spleen, and liver (but not in the lungs or small intestine) at 5 h after the onset of sepsis. These results indicate that the heart, spleen, and liver appear to be important ET-producing organs during the early stage of sepsis. The lack of significant increases in tissue ET levels could be due to the possibility that the newly converted peptide is quickly transferred to the bloodstream. Since the hyperdynamic phase of sepsis occurs at 2–10 h and the hypodynamic phase occurs at 20 h after CLP, the increased plasma levels of ET at 5 and 10 h suggest that mediators other than ETs (such as adrenomedullin) are responsible for producing the biphasic hemodynamic responses during the progression of polymicrobial sepsis.

Keywords: Hyperdynamic sepsis; Hypodynamic sepsis; Cecal ligation and puncture

1. Introduction

While therapeutic approaches for the management of sepsis have advanced in recent years, the incidence of septic shock has increased significantly over the past 20 years, and it remains the leading cause of death among surgical intensive care patients [1,2]. There are two distinct phases associated with polymicrobial sepsis. The early, hyperdynamic phase is characterized by increased cardiac output and tissue perfusion and decreased vascular resistance, and the later, hypodynamic phase is characterized by reduced

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cardiac output and tissue perfusion [2,3]. A recent study by Szalay et al. has indicated that endothelins play a role in producing the hypodynamic response during sepsis by showing that blockade of endothelin$_A$ (ET_A) receptors attenuates the increased total peripheral resistance and decreased cardiac output observed during hypodynamic sepsis [4]. If ET_A receptor binding does in fact contribute to the hemodynamic response seen during hypodynamic sepsis, one would expect an increase in circulating levels of the ligand (i.e. ET-1 and ET-2, potent vasoconstrictors) under such conditions. We tested the hypothesis that upregulation of ETs closely coincides with the initiation of the hypodynamic phase of sepsis by determining at what time points circulating levels of ETs are increased. In addition, recent studies have shed light on the relationship between ETs and other known mediators of sepsis, including TNF-α and IL-1β [5–8]. These cytokines have been shown to upregulate ET-1 gene expression in various cell populations [6,7].

Endothelins belong to a family of bicyclic 21-amino acid residue peptides, which also includes sarafotoxins, mouse vasoactive intestinal contractor, and bibrotoxin. Three ET isopeptides (ET-1, ET-2, and ET-3) are the most potent known vasoconstrictors of vascular smooth muscle, and their pharmacological roles in various tissue beds are well documented [9–12]. The strong vasoactive properties of ETs have led to speculation that they might play an integral role in the hemodynamic changes associated with sepsis syndrome. ET-1 was first isolated from porcine aorta endothelial cells in 1988 by Yanagisawa et al. [9]. Shortly after, ET-2 and ET-3 were sequenced [13]. Although ET isopeptides possess a highly conserved primary structure, they differ in their receptor selectivity and biological activities [14–16]. In this regard, ET-1's affinity for ET_A receptors is 8 times as strong as ET-2's, and 1000 times as strong as ET-3's. Since ET_A receptors primarily mediate vasoconstriction, ET-1 is therefore considered the predominant vasoconstrictive isopeptide. The three isopeptides show similar potency for ET_B receptors, which elicit both vasoconstriction and vasodilation, depending on their localization. Karne et al. have cloned an ET-3-selective receptor, termed ET_C, from *Xenopus laevis*, though a mammalian source has yet to be found [17]. Since ET-1's potency is similar to or greater than the others, it appears that ET-1 is the predominant isopeptide during sepsis. Endothelins are produced by a three-stage synthetic pathway. Transcription of the ET genes yields 203–212-amino acid residue proteins called preproendothelins, which are cleaved by specific endopeptidases to form big ETs. Final conversion of these precursors into mature ETs is accomplished by proteases known as ET converting enzymes.

Circulating levels of ETs have been shown to increase in various cardiovascular, renal, and respiratory disorders, all of which are associated with damage to the vascular endothelium [18]. Plasma levels of ET-1 and ET-3 are elevated in septic patients [19–24], and the levels of ETs are positively correlated with the severity of the illness [24]. It has been suggested that the release of ETs during sepsis has both beneficial and detrimental effects. While their vasoconstricting effects may counteract the general hypotension and decreased cardiac output associated with septic shock, ETs also contribute to the increased vascular resistance that can eventually lead to multiple organ failure. Alterations in plasma and tissue concentrations of ETs have been well documented during various endotoxemia models [25–27]. Few studies, however, have employed the cecal ligation and puncture (CLP) model of sepsis, with its clearly defined hyperdynamic and hypodynamic phases, to examine the role ETs during polymicrobial sepsis. The aim of this study, therefore, was to determine at which time point plasma endothelin concentrations are increased and which organs contribute to increased endothelin levels during polymicrobial sepsis.

2. Materials and methods

2.1. Animal model of polymicrobial sepsis

Male Sprague–Dawley rats (275–325 g) were subjected to polymicrobial sepsis by CLP [28]. Briefly, rats were fasted overnight, but allowed water ad libitum. After anesthetization with methoxyflurane inhalation, a 2-cm ventral midline incision was made, and the cecum was exposed and ligated just distally to the ileocecal valve. The cecum was then punctured twice with an 18-gauge needle, squeezed slightly to

allow a small amount of fecal matter to flow from the holes, and then returned to the abdominal cavity. The incision was closed in layers and the wound was bathed in 1% lidocaine solution to provide analgesia. The animal received a subcutaneous injection of normal saline (3 ml/100 g body weight) immediately after CLP (i.e. fluid resuscitation). Sham-operated animals were subjected to the same procedure though the cecum was neither ligated nor punctured. The experiments described herein were performed in adherence to the National Institutes of Health guidelines for the use of experimental animals. This project was approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital (Providence, RI).

2.2. Determination of plasma and tissue levels of ETs

Blood samples were drawn at 2, 5, 10, and 20 h following CLP or sham-operation by cardiac puncture under methoxyflurane anesthesia ($n = 8$–10 rats at each time point). Samples were also collected from a normal control group which underwent no operation. Because multiple sampling may significantly affect blood pressure and cardiac output, repeated blood samples were not taken from the same animal at different time points. Blood was drawn into a heparinized syringe, immediately placed on ice, and then centrifuged at 3000 rpm at 4°C for 10 min using a Sorvall RT6000D centrifuge (DuPont, Hoffman Estates, IL). Plasma was frozen in 1.5-ml aliquots and stored at −80°C until assay. Plasma levels of ET-1, ET-2, and big ET-1 were measured using a specific radioimmunoassay kit (Endothelin-1,2 (high sensitivity) (^{125}I) assay system, Amersham, Arlington Heights, IL). Briefly, ETs were extracted from acidified plasma samples using Amprep 500 mg C2 columns (Amersham, Arlington Heights, IL) and eluted with 80% acetonitrile and 0.1% trifluoroacetic acid. The eluant was then evaporated overnight in a centrifugal concentrator (Savant Speed Vac Plus SC110A, Savant Instruments, Farmingdale, NY), reconstituted in assay buffer, and analyzed according to the manufacturer's protocol. In additional groups of animals, the heart, lungs, small intestine, and spleen were harvested at 5 h after CLP or sham-operation and snap frozen in liquid nitrogen. The tissue samples were then homogenized in 25% 2 M HCl, centrifuged at 3000 rpm for 30 min, and the supernatant was frozen at −80°C. ETs were extracted and assayed as described above.

2.3. Reverse-transcription polymerase chain reaction (RT-PCR)

Heart, liver, spleen, lung, and small intestine samples were harvested at 5 h after CLP or sham-operation. Total RNA was extracted using Tri-reagent (Molecular Research Center, Cincinnati, OH) and 4 μg of RNA was reverse transcribed as previously described by us [29]. The resulting cDNAs were amplified by polymerase chain reaction (PCR) using specific primer for rat preproendothelin-1 (preproET-1, sense, 5′-GCTCCAGAAACAGCTGTCTTGGGA-3′; antisense, 5′-GGAGGTCTTGATGCTGTTGCTGA-3′) [30]. Rat glyceraldehyde 3-phosphate dehydrogenase (G3PDH) served as a housekeeping gene (Clontech, Palo Alto, CA). PCR cycling proceeded as follows: 94°C for 1 min, 58°C for 2 min, and 72°C for 3 min. Heart, small intestine, and liver samples were amplified with 35 cycles, lung and spleen samples with 30 cycles, and G3PDH samples with 25 cycles. Following the RT-PCR procedure, the PCR amplification products were electrophoresed using a 1.6% agarose gel containing 0.22 μg/ml ethidium bromide. The gel was then photographed using Polaroid film.

2.4. Statistical analysis

Results are presented as means ± S.E.M. One-way analysis of variance and Tukey's test or unpaired Student's *t*-test was used and the differences were considered significant at $P \leq 0.05$.

3. Results

3.1. Alterations in plasma ET levels

Alterations in plasma levels of ETs (the sum of ET-1, ET-2, and big ET-1, which cannot be separately measured by the current RIA) are summarized in Fig. 1. At 2 h after CLP, there were no significant increases in plasma ETs. At 5 and 10 h after CLP, however, septic animals showed approximately a

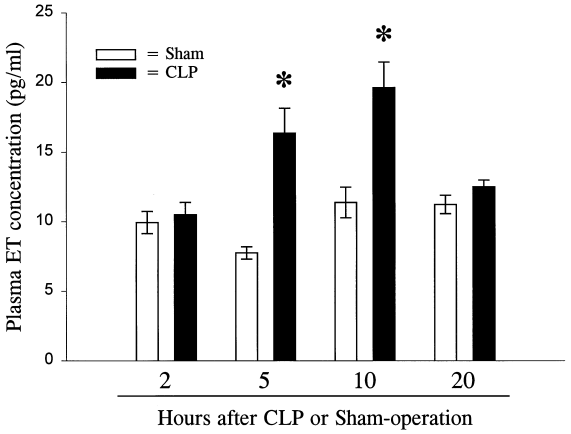

Fig. 1. Plasma levels of endothelins at various time-points after cecal ligation and puncture (CLP) or sham operation (Sham). The data are presented as means ± S.E.M.($n = 7$–10/group) and compared by one-way analysis of variance and Tukey's test. *$P < 0.05$ versus the respective sham-operated animals.

two-fold increase as compared to sham-operated and normal rats ($P < 0.05$, 16.4 ± 1.74 versus 7.7 ± 0.5 pg/ml at 5 h, and 19.7 ± 1.7 versus 11.56 ± 1.0 pg/ml at 10 h; plasma levels in normal rats were 11.7 ± 1.2 pg/ml). At 20 h after CLP, concentrations of plasma ETs returned to sham levels (Fig. 1).

3.2. Alterations in tissue ET levels

As indicated in Fig. 2, cardiac ET levels in septic animals were not significantly different from sham-operated animals. Similarly, there were no significant alterations in ET levels in the lungs, small intestine, or spleen at 5 h after the onset of sepsis.

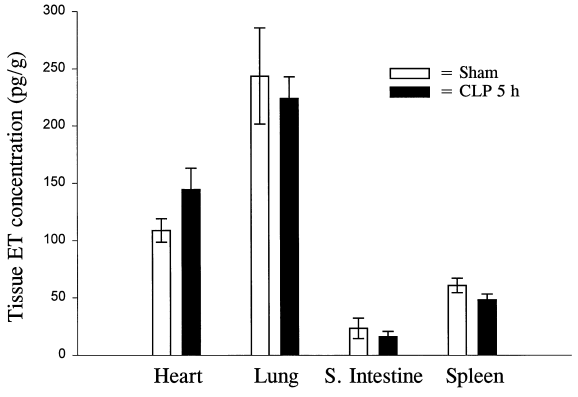

Fig. 2. Tissue levels of endothelins at 5 h after cecal ligation and puncture (CLP) or sham operation (Sham). The data are presented as means ± S.E.M. ($n = 5$–7/group) and compared by unpaired Student's t-test. *$P < 0.05$ versus the respective sham-operated animals.

3.3. Alterations in preproET-1 gene expression

The RT-PCR results in Fig. 3C indicate that preproET-1 gene expression increased in the heart (lane 3, 488 bp) and liver (lane 7) at 5 h after the onset of

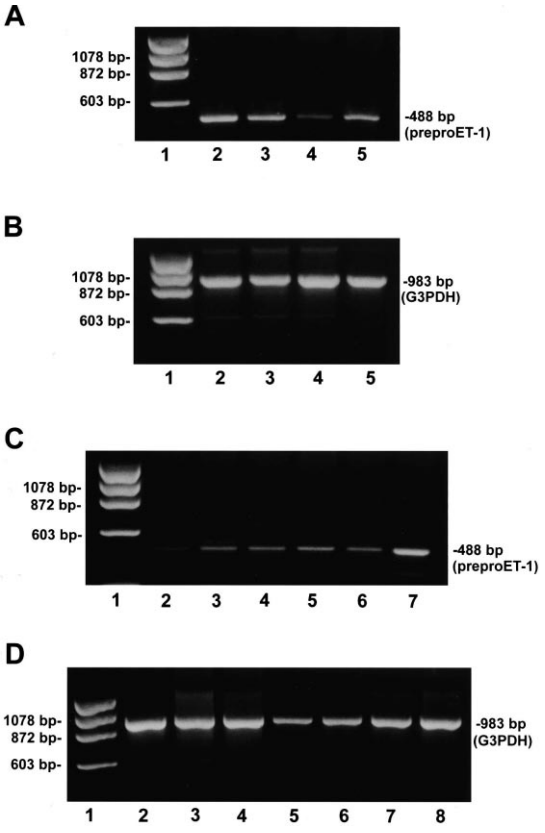

Fig. 3. Gene expression of preproendothelin-1 (488 bp) (A,C) as well as the housekeeping gene G3PDH (983 bp) (B,D) in the lungs, spleen, heart, small intestine, and liver at 5 h after cecal ligation and puncture or sham operation. (A) Lane 1, X174/*Hae*III size markers; lane 2, lung preproET-1 in sham animals; lane 3, lung preproET-1 in CLP animals; lane 4, splenic preproET-1 in sham animals; lane 5, splenic preproET-1 in CLP animals. (B) Lane 1, X174/*Hae*III size markers; lane 2, lung G3PDH in shams; lane 3, lung G3PDH in CLPs; lane 4, splenic G3PDH in shams; lane 5, splenic G3PDH in CLPs. (C) Lane 1, X174/*Hae*III size markers; lane 2, cardiac preproET-1 in shams; lane 3, cardiac preproET-1 in CLPs; lane 4, intestinal preproET-1 in shams; lane 5, intestinal preproET-1 in CLPs; lane 6, hepatic preproET-1 in shams; lane 7, hepatic preproET-1 in CLPs. (D) The order of each lane is identical to that in C, with the exception that lane 2 here is a G3PDH positive control.

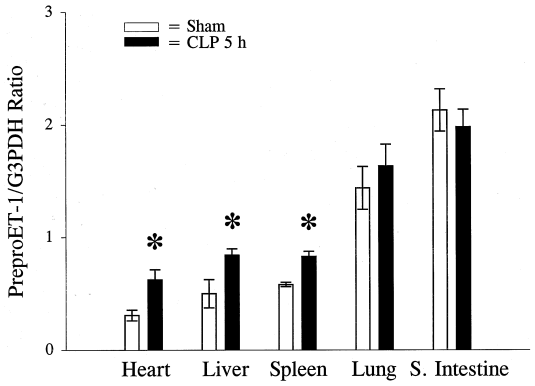

Fig. 4. The intensity ratio of preproendothelin-1 gene versus housekeeping gene G3PDH in various organs at 5 h after cecal ligation and puncture (CLP) or sham operation (Sham). The data are presented as means ± S.E.M. ($n = 3$–5/group) and compared by unpaired Student's t-test. $^*P < 0.05$ versus the respective sham-operated animal.

sepsis as compared to sham-operated animals (lanes 2 and 6, respectively). Similarly, preproET-1 mRNA levels increased significantly in the spleen (lane 5, Fig. 3A) at 5 h as compared to shams (lane 4, Fig. 3A). Fig. 3B and D indicate that expression of the housekeeping gene G3PDH (983 bp) was similar in both sham and CLP groups in all of the tissues tested, indicating that similar levels of reverse transcription were achieved. Optical density of target and housekeeping gene bands were determined by Mocha Image Analysis and Sigma Gel software. PreproET-1/G3PDH band density ratios were calculated. The results indicate that the preproET-1/G3PDH ratio increased significantly in the heart, liver, and spleen, but not in the lungs and small intestine at 5 h after the onset of sepsis (Fig. 4).

4. Discussion

Endothelin-1 is the most potent known vasoconstrictor released by vascular endothelial cells [9–11]. As such, members of the ET family of isopeptides, in conjunction with eicosanoids and other vasoactive substances such as nitric oxide (NO), are thought to play a key role in regulating hemodynamic responses that take place during various disease states, including sepsis [24,31–34]. There is some controversy, however, as to whether the role ETs is beneficial or detrimental. Wanecek et al. reported that administration of the mixed non-peptide ET receptor antagonist bosentan counteracted the cardiac dysfunction seen during endotoxemia by reducing systemic vascular resistance and improving stroke volume index and systemic oxygen delivery [35]. Conversely, Ruetten et al. administered the non-selective ET receptor antagonist SB 209670 after endotoxin infusion and reported an exacerbation of hypotension, vascular hyporeactivity to norepinephrine, renal dysfunction, and hepatocellular injury as compared to rats receiving endotoxin alone [36]. In contrast to endotoxin, the role of ETs in the progression of polymicrobial sepsis remains unclear.

Although studies have documented deviations in plasma and tissue concentrations of ETs following endotoxin injection or in a cecal-slurry model of sepsis [25–27], little research has been conducted on this subject using the CLP model of polymicrobial sepsis. This model of sepsis closely mimics the hemodynamic changes that occur in the usual clinical arena, where an early, hyperdynamic phase (characterized by increased tissue perfusion and cardiac output, and decreased total peripheral resistance) is followed by a late, hypodynamic phase (characterized by reduced cardiac output and tissue perfusion) [3]. The studies of Szalay et al. suggest that ET-1 might play a role in producing the hypodynamic phase by showing that ET-A receptor blockade attenuates the increased total peripheral resistance and decreased cardiac output seen during hypodynamic sepsis [4]. Little is known, however, about what role, if any, ETs play in producing the hypodynamic phase of polymicrobial sepsis, or the transition from the hyperdynamic response to the hypodynamic response of sepsis. The purpose of the present study, therefore, was to determine at which time point plasma ET concentrations are increased and which organs contribute to increased ET levels during polymicrobial sepsis.

Our results indicate that plasma levels of ETs (ET-1, ET-2, and big ET-1) were significantly increased at 5 and 10 h after CLP, but remained close to sham levels at 2 and 20 h after CLP. The lack of the increase in plasma concentrations of ETs during the late stage of sepsis is consistent with the findings of other investigators using cecal slurry and LPS infusion models in rats and sheep [26,27]. In contrast, the increased ET levels appear to occur much earlier

following endotoxemia [37]. Since LPS infusion has been shown to induce pulmonary hypertension and increased vascular resistance in anesthetized animals [38], direct administration of endotoxin may produce a much earlier upregulation of ETs, which appears to be responsible for intense vasoconstriction. This discrepancy between the models is most likely due to the accelerated nature of the endotoxin model, which lacks the distinct phases seen in the CLP model of sepsis. As a result, direct administration of endotoxin may produce a much earlier upregulation of ETs.

To determine the source of the upregulated ETs, tissue levels of ETs were determined in the heart, lung, small intestine, and spleen at 5 h after CLP. Though there was a 33% increase in cardiac levels of ETs, there were no significant changes in any of the tissues tested. A few studies have been conducted to measure tissue levels of ETs during sepsis. Sharma et al. reported that ET levels in the left ventricle of septic rats were significantly increased only at 12 h following the induction of sepsis by cecal slurry, as compared to non-septic rats [26]. We propose two explanations for the lack of significant increases in tissue levels of ETs at 5 h after the onset of sepsis. First, as reported by Lundberg et al., although ETs appear to be metabolically stable in plasma (with a half-life of 1–2 min), major local tissue degradation (ranging from 50–90%) represents a problem when attempting to measure tissue ET levels [39]. Secondly, it is possible that the newly synthesized ETs are quickly released into the bloodstream after conversion into active peptides, making it difficult to detect the true increase in tissue levels of ETs during the course of sepsis. To further investigate the sources of the upregulated ETs detected in plasma, RT-PCR was performed to detect preproET-1 gene expression changes at 5 h after CLP in the heart, lung, spleen, small intestine, and liver. The significant upregulation observed in the heart, liver, and spleen, which is consistent with earlier studies [25,40–42], suggests that these organs are important sources of the upregulated ETs seen during the early phase of sepsis. It should be pointed out that only preproET-1 mRNA was assessed in the present study. This was done since ET-1 appears to be the predominant isopeptide in producing vasoconstriction under various adverse circulatory conditions.

Polymicrobial sepsis is characterized by an early, hyperdynamic phase followed by a late, hypodynamic phase. We have previously demonstrated that the hyperdynamic response occurs as early as 2 h after CLP [3,43]. Since ET production is not upregulated earlier than 5 h after the onset of sepsis, it does not appear that ETs play a major role in producing the hyperdynamic response in early sepsis. It could be argued that preproET-1 gene upregulation begins earlier than 5 h after CLP, leading to the production of mature ET-1 that can act in a paracrine fashion before it can be detected in the bloodstream. This possibility is unlikely since locally increased ET levels should be detected in tissue measurements, but such increases were absent, even at 5 h post-CLP. Our recent studies suggest that upregulation of the potent vasodilatory peptide adrenomedullin (ADM), rather than ETs, might play an integral role in producing the hyperdynamic phase associated with early polymicrobial sepsis [29]. When normal rats were infused with synthetic rat ADM at a dose which does not significantly affect blood pressure, they displayed increased cardiac output, stroke volume, and microvascular blood flow in various organs and decreased peripheral resistance [44]. In addition, the hyperdynamic and hypercardiovascular responses normally observed during early sepsis were prevented with administration of anti-ADM antibodies [44].

Cytokines such as TNF-α and IL-1β have been shown to upregulate preproET-1 expression in various types of isolated cells [5–8]. Conversely, NO, whose production during late sepsis is augmented due to iNOS upregulation [45], downregulates ET-1 production [46]. This late increase in plasma NO provides one explanation for the return of plasma ET levels to sham levels during the later stages of sepsis. It is possible that a delicate balance between these vasoregulators is disrupted during sepsis, leading to the diminished vascular tone, peripheral hypoperfusion, and multiple organ failure associated with septic shock.

In summary, our results demonstrate that circulating levels of ETs were not elevated at 2 or 20 h, but were significantly increased at 5 and 10 h after CLP. While no significant increases in tissue levels were detected at 5 h, preproET-1 gene expression was upregulated in heart, spleen, and liver at 5 h after CLP. Since our previous studies have shown that the hyperdynamic phase of sepsis begins as early as 2 h

after CLP, and since ET production is not upregulated earlier than 5 h, we propose that factors other than ETs are responsible for producing the transition from the hyperdynamic to the hypodynamic response during the progression of polymicrobial sepsis.

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